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Factors affecting neuronal birth and death in the mammalian olfactory epithelium

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Abstract. To identify factors regulating neurogenesis and neuronal death in mammals and to determine the mechanisms by which these factors act, we have studied mouse olfactory epithelium using two different experimental paradigms: tissue culture of olfactory epithelium purified from mouse embryos; and ablation of the olfactory bulb in adult mice, a procedure that induces olfactory receptor neuron (ORN) death and neurogenesis *in vivo*. Studies of olfactory epithelium cultures have allowed us to characterize the cellular stages in olfactory neurogenesis and to identify factors regulating proliferation and differentiation of precursor cells in the ORN lineage. Studies of adult olfactory epithelium have enabled us to determine that all cell types in this lineage—proliferating neuronal precursors, immature ORNs and mature ORNs—undergo cell death following olfactory bulb ablation and that this death has characteristics of programmed cell death or apoptosis. *In vitro* studies have confirmed that neuronal cells of the olfactory epithelium undergo apoptotic death and have permitted identification of several polypeptide growth factors that promote survival of a fraction of ORNs. Using this information, we have begun to explore whether these factors, as well as genes known to play crucial roles in cell death in other systems, function to regulate apoptosis and neuronal regeneration in the adult olfactory epithelium following lesion-induced ORN death.

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The number of neurons in a nervous system ultimately determines its complexity. In the vertebrate nervous system, this number is determined during development and is primarily regulated by two processes: neurogenesis

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and naturally occurring cell death. The focus of our work has been to identify the molecules and cell interactions that control these two processes during the development of the mammalian nervous system.

In mammals, neurogenesis, the proliferation of neuronal precursor cells and differentiation of their progeny into postmitotic neurons, occurs primarily during embryonic development. Neurogenesis is both spatially and temporally regulated (e.g. Oppenheim et al 1989), but the factors involved in this regulation have only recently begun to be understood. Several investigators have localized the expression of genes encoding polypeptide growth factors and their receptors to regions in or near the embryonic nervous system, suggesting that these molecules are involved in regulating proliferation and/or differentiation of neuronal precursor cells (e.g. Maisonnier et al 1990, Schecterson & Bothwell 1992, Orr-Urtreger et al 1991). Together with studies demonstrating effects of polypeptide growth factors on both proliferation and survival of neuronal precursor cells *in vitro* (e.g. DiCicco-Bloom et al 1993, Birren et al 1993, DeHamer et al 1994), this indicates that growth factors play an important role in regulating neurogenesis, and therefore neuron number, during nervous system development.

A large fraction of neurons die as development proceeds. In some regions, more than half of neurons die during the course of this naturally occurring cell death (Oppenheim 1991). Survival of developing neurons appears largely to be dependent upon them obtaining adequate trophic support and, in many instances, this support is provided by polypeptide growth factors produced by cells in the synaptic target field (reviewed in Barde 1989). The cell death neurons undergo when denied adequate trophic support displays distinctive biochemical features, in particular a requirement for *de novo* gene expression. This is demonstrated by the ability of transcriptional and translational inhibitors to rescue trophic factor-dependent neurons from death when factors are withdrawn (e.g. Martin et al 1988). This type of cell death, referred to as programmed cell death or apoptosis, appears to play an important role in matching neuron number to target size during nervous system development (Oppenheim 1991, Johnson & Deckwerth 1993).

The mammalian olfactory epithelium is uniquely suitable as a model system for studying the regulation of neuron number in the mammalian nervous system. In the olfactory epithelium, proliferation of neuronal precursor cells, differentiation of their progeny into olfactory receptor neurons (ORNs) and turnover of ORNs are processes that begin during embryonic development and appear to continue throughout the lifetime of the organism (Graziadei & Monti Graziadei 1978, 1979). There is also evidence that cell interactions regulate both neurogenesis and neuronal death in the olfactory epithelium: ORN death can be up-regulated in the adult mammal by lesioning the axons of ORNs or ablating their synaptic target tissue, the main olfactory bulb of the brain. Such operations cause degeneration of ORNs and result in increased

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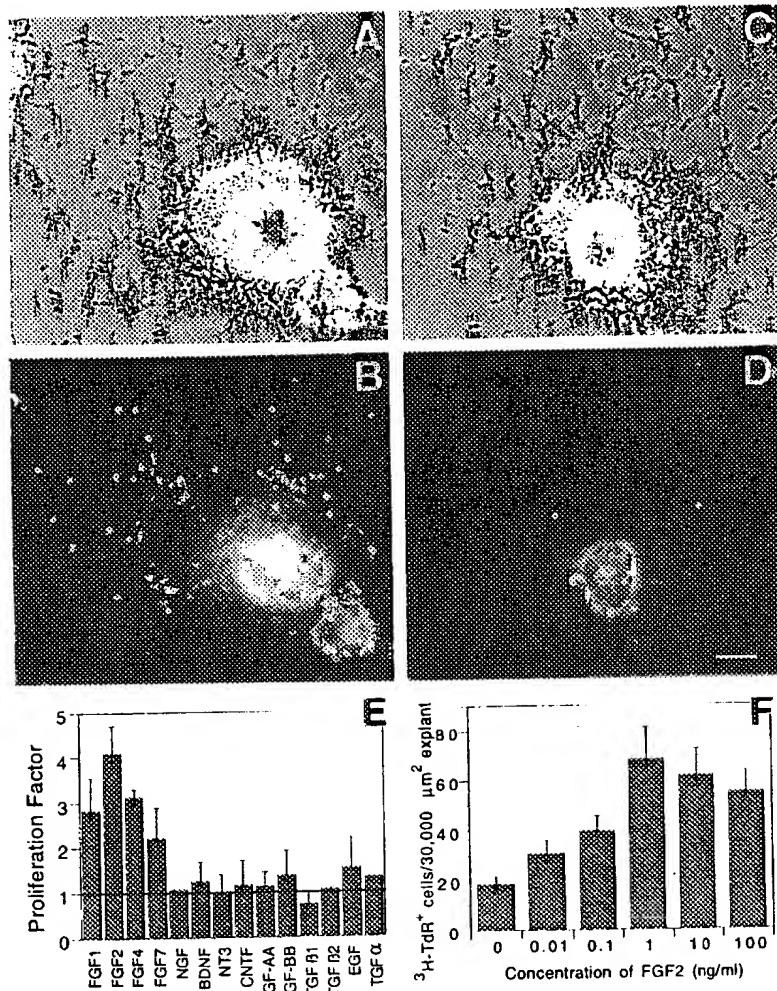
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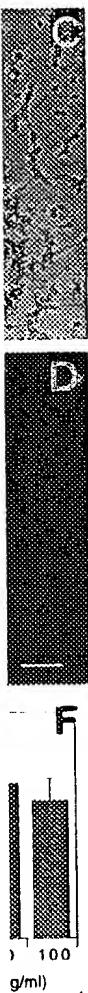


mitotic activity in the neuronal precursor cells of the epithelium, leading to the production of new ORNs (Monti Graziadei & Graziadei 1979, Costanzo & Graziadei 1983, Schwartz Levey et al 1991, Holcomb et al 1995). Thus, the properties of the mammalian olfactory epithelium provide a unique opportunity to study, in a single tissue, how neurogenesis and neuronal death interact to control neuron number in the adult, as well as the embryonic, mammal.

Factors regulating the genesis of olfactory receptor neurons

Our initial studies of the genesis of ORNs in embryonic mouse olfactory epithelium explant cultures indicated that this process proceeds through four distinct cell stages: (1) a stem cell was thought to give rise to (2) the immediate neuronal precursor (INP) of ORNs. In minimally supplemented, defined medium, the INP divides to generate (3) two daughter cells—immature, postmitotic ORNs—which begin to express the neural cell adhesion molecule (NCAM) within about 12 hours of this terminal division (Calof & Chikaraishi 1989). *In vivo*, these ORNs subsequently synapse upon neurons of the olfactory bulb and express markers characteristic of (4) mature ORNs.

FIG. 1. FGFs promote late-occurring neurogenesis in olfactory epithelium cultures. Olfactory epithelium was isolated from embryonic day 14.5–15.5 CD-1 mouse embryos and olfactory epithelium explants were cultured in serum-free, defined medium on glass coverslips coated with poly-D-lysine and merosin as described (DeHamer et al 1994). Cultures were grown with indicated growth factors continuously present, incubated with [³H]thymidine (0.1 μ Ci/ml) from times 24 to 48 h in culture, fixed and processed for autoradiography. (A) Phase contrast, FGF-2-treated culture (10 ng/ml). (B) Same field as (A), darkfield. (C) Phase contrast, control (no growth factor). (D) Same field as (C), darkfield. (Bar = 50 μ m.) (E) Growth factor screening assays. Bars represent mean proliferation factor \pm SD for growth factors at optimum tested concentrations (FGF-1, 100 ng/ml; FGF-2, 1 ng/ml; FGF-4, 10 ng/ml; FGF-7, 10 ng/ml; NGF, BDNF and NT3, 50 ng/ml; CNTF, PDGF-AA and -BB, and TGF- β 1 and β 2, 10 ng/ml; EGF and TGF- α , 20 ng/ml). FGFs 4 and 7 were each also tested at 1 and 100 ng/ml, and gave effects similar to those observed at 10 ng/ml (not shown). Proliferation factors were calculated as follows: for each explant, the *explant labelling index* was calculated as the number of migratory cells with silver grains over their nuclei divided by the area of the explant (measured using NIH Image 1.52). The *proliferation factor* is the ratio of the mean *explant labelling index* in a given condition to that of controls (no added growth factor) grown on the same day. Percentage error for these ratios (the square root of the sum of the squares of percentage errors [from SEM] of the two labelling indices being compared) averaged ~20%. Analyses of variance followed by Dunnett's test (for multiple comparisons against a single control) were performed for every experiment. No growth factors other than the FGFs were found to have statistically significant effects ($P < 0.05$) on *explant labelling index*. (F) Dose-response analysis for FGF-2. Points represent mean \pm SEM for *explant labelling index* at each concentration. The unit of area used in normalizing the *explant labelling indices* was 30 000 μ m², the approximate mean *explant area* for all tests. (Reproduced with permission from DeHamer et al 1994.)



In vitro, neurogenesis ceases after about 24 hours in olfactory epithelium explants cultured in the absence of exogenous growth factors. *In vivo*, however, neurogenesis in the olfactory epithelium continues throughout the lifetime of the organism. To determine whether it is possible to restore continual neurogenesis to olfactory epithelium cultures, we initiated a series of experiments to screen for growth factors that could promote 'late' neurogenesis (i.e. neurogenesis occurring after 24 hours *in vitro*) in cultured olfactory epithelium explants. The results of these screening assays (illustrated in Fig. 1) indicated that four members of the fibroblast growth factor (FGF) family—FGF-1, FGF-2, FGF-4 and FGF-7—were able to promote a significant level of 'late' neurogenesis. In contrast, neurotrophins, ciliary neurotrophic factor, platelet-derived growth factors, transforming growth factor β s and members of the epidermal growth factor family were unable to promote extended neurogenesis in olfactory epithelium explant cultures (Fig. 1E). A more detailed analysis of the mechanism of FGF-2's effects indicated that FGFs act on INPs to increase the likelihood that these cells will divide twice, rather than once, in culture. However, the ultimate neuronal fate of INPs is unaffected when they are grown in the presence of FGFs (DeHamer et al 1994). Thus, in addition to identifying FGFs as growth factors that act to promote neurogenesis in the olfactory epithelium, these studies have contributed to our concept of the developmental stages of the ORN lineage: the INP appears to function as a neuronal transit amplifying cell, undergoing a limited number of divisions (which can be regulated by FGFs) before its progeny undergo requisite differentiation to become ORNs.

In addition to regulating INP division, FGFs also appear to have effects on progenitor cells at an earlier stage in the ORN lineage. In FGF-treated cultures, rare explants with proliferating cells that continue to generate ORNs can be found at 72 and 96 hours *in vitro*. The incidence of these explants (5–8% of total explants continue to exhibit this property at late times in culture) is consistent with the view that an early, rare progenitor is present in only a small fraction of olfactory epithelium explants, and that FGF supports the proliferation and/or survival of this cell (DeHamer et al 1994). Our current hypothesis is that this cell is the stem cell ultimately responsible for continual neurogenesis in the olfactory epithelium, although this has not yet been demonstrated directly.

The similarity of INPs to transit amplifying cells in other tissues raises the possibility that earlier, distinct stages of amplifying precursor cells, in addition to INPs, might exist in the ORN lineage. One clue as to the identity of such cells has come from our recent studies of mice (generated by F. Guillemot and colleagues) in which the gene encoding the transcription factor MASH-1 (mammalian achaete-scute homologue 1) has been inactivated by homologous recombination. In these mice, there is a profound loss of ORNs, as well as many autonomic and enteric neurons (Guillemot et al 1993). In order to determine whether MASH-1 is acting in cells of the ORN lineage, and at what stage, we have investigated the dynamics of MASH-1 expression during

epithelium, however, the time of the neurogenesis to screen for neurogenesis in explants. We found that four GDF-2, FGF-4, and EGF promote growth in olfactory epithelium. In addition to growth factors, the mechanism of growth is likely to involve the presence of growth factors as growth stimuli, these being the source of the ORN amplifying cell, which is stimulated by FGFs) and by FGFs)

neurogenesis on cultured cultures, ORNs can be ~8% of total. This is consistent with the fraction of proliferation and/or the fact that this cell is the olfactory

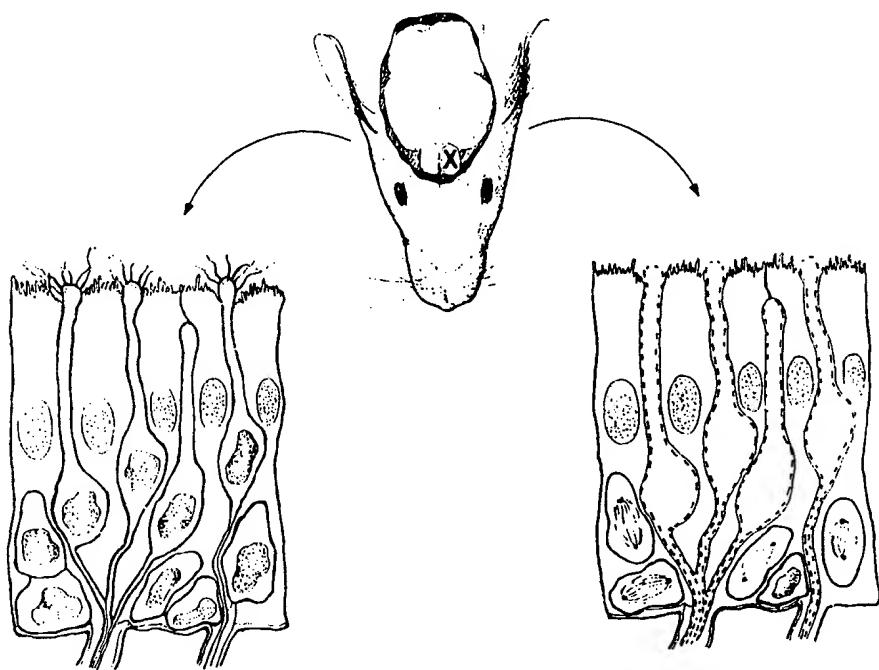


FIG. 2. Unilateral olfactory bulbectomy and its effects on the ipsilateral olfactory epithelium. Adult male mice are anaesthetized and a small suction tube is used to selectively remove the left olfactory bulb without causing injury to the contralateral olfactory bulb or to the brain (Holcomb et al 1995). The 'X' in the figure marks the ablated left olfactory bulb. The arrows point to cartoons of contralateral olfactory epithelium (mouse's right), and olfactory epithelium deprived of its target tissue (bulbectomized olfactory epithelium; mouse's left). Compared with the unaffected olfactory epithelium on the contralateral side, most olfactory receptor neurons (ORNs) die in bulbectomized olfactory epithelium, and vacant spaces left by degenerated ORNs are readily apparent. Mitotic figures in cells of the bulbectomized olfactory epithelium indicate that proliferation of neuronal precursors, located in the basal compartment of the epithelium, is up-regulated following olfactory bulbectomy.

This raises the question, in addition to the identity of such a gene, whether MASH-1 homologous genes, as well as other genes, are involved. In order to address this question during

we have characterized neurogenesis in the olfactory epithelium in two different paradigms: *in vitro*, in olfactory epithelium derived from the mouse embryo, and *in vivo*, during neurogenesis induced by unilateral ablation of the olfactory bulb (olfactory bulbectomy, OBX). Our results indicate that MASH-1-expressing cells are not stem cells, but instead are progenitors of INPs, and like them, function as neuronal transit amplifying cells in the ORN lineage (Calof et al 1994, 1995, Gordon et al 1995). To facilitate our study of the molecular mechanisms that regulate developmental progression of ORN progenitor cells, we have begun characterizing early progenitor cells that are isolated from olfactory epithelium cultures by immunological panning (Mummery et al 1996). We have also

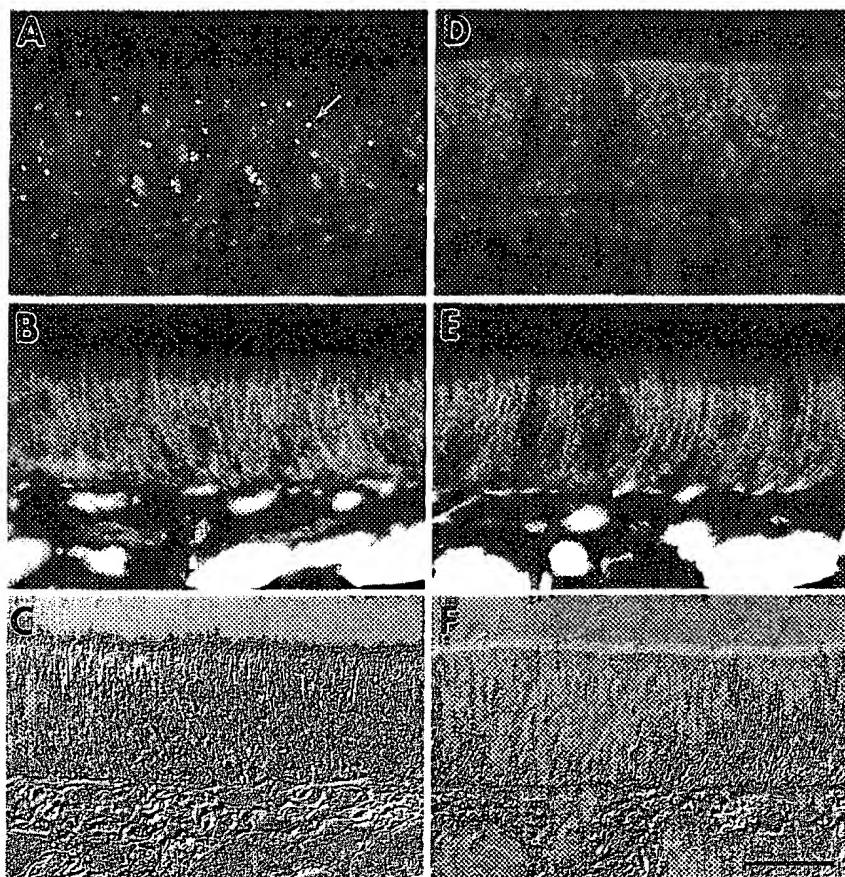


FIG. 3. DNA fragmentation in the olfactory epithelium following unilateral olfactory bulbectomy. Adult male mice were sacrificed at time points ranging from 12 hours to 84 days post-bulbectomy. The region of the nose containing the olfactory epithelium was dissected and fixed by freeze substitution. After decalcification for 7 days in $\sim 390\text{ mM}$ EDTA, pH 7.1, the tissue was sectioned in the horizontal plane in $12\text{ }\mu\text{m}$ sections with a cryostat. Sections of olfactory epithelium were stained for DNA fragmentation using deoxynucleotide terminal transferase end-labelling of DNA fragments with biotinylated dUTP and a fluorescent avidin, a modification of the TUNEL technique of Gavrieli et al (1992). Shown are the bulbectomized epithelium (A-C) and contralateral unoperated epithelium (D-F) immediately opposite, from an animal sacrificed at 24 hours post-bulbectomy. (A, D) Fluorescein optics showing TUNEL staining; arrow in A indicates TUNEL-positive cell. (B, E) Rhodamine optics showing NCAM-immunoreactivity in the same sections. (C, F) Nomarski optics. (Bar = $50\text{ }\mu\text{m}$.)



generated immortalized cell lines that share morphological and molecular similarities to these early progenitor cells (Hagiwara et al 1995). Interestingly, some of these cell lines express MASH-1, and should therefore be useful for looking at how expression of this transcription factor provides a crucial step in ORN development (Hagiwara et al 1995).

Thus, our current view of the ORN lineage is as follows: (1) a rare, self-renewing stem cell (for which no molecular markers are yet available) generates (2) a MASH-1-expressing progenitor cell. The MASH-1-expressing progenitor cell gives rise to (3) INPs, which do not express MASH-1 or NCAM, and which undergo amplification divisions in response to FGF. Ultimately, the progeny of INP divisions differentiate to become (4) postmitotic ORNs. These terminally differentiated neurons undergo further development to become (5) mature ORNs following contact with their synaptic target tissue, the olfactory bulb.

Factors regulating survival and apoptosis of neuronal cells of the olfactory epithelium

Our first goal in these studies has been to characterize the timecourse and extent of cell death in the olfactory epithelium following removal of the olfactory bulb, and to determine if this death has the characteristics of apoptosis. For these experiments, we surgically ablate the left olfactory bulb of adult male mice (Fig. 2). These bulbectomized mice are then sacrificed at different post-surgery timepoints, ranging from hours to weeks. We have used fragmentation of nuclear DNA as an *in situ* indicator of apoptosis. To detect apoptotic cells, cryostat sections of olfactory epithelium from OBX mice are treated with deoxynucleotide terminal transferase and dUTP biotin to end-label fragmented DNA. The incorporated biotin is then detected using a fluorescent avidin (modification of the TUNEL method of Gavrieli et al 1992).

Figure 3 shows an example of this in sections from an animal sacrificed 24 hours after OBX. In (A) TUNEL staining in olfactory epithelium from the OBX side is shown; the white dots (arrow) are the nuclei of cells with fragmented DNA. It is clear that many cells are already undergoing DNA fragmentation—and by extension, apoptotic cell death—by 24 hours after OBX. Double-staining of this section with an antibody to NCAM (B), which detects all postmitotic ORNs (Calof & Chikaraishi 1989, Holcomb et al 1995), demonstrates that the vast majority of cells undergoing apoptosis are ORNs. In contrast, there is virtually no TUNEL staining in the olfactory epithelium on the contralateral (unoperated) side of the same animal (D), and the complement of NCAM-positive cells is essentially the same (E). It is interesting to note that a high level of TUNEL staining is evident prior to any overt signs of morphological degeneration in the olfactory epithelium (compare C with F), indicating that DNA fragmentation precedes overt cell loss during the course of apoptosis.

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TABLE 1 Temporal analysis of DNA fragmentation following unilateral olfactory bulbectomy

Time (days)	TUNEL-positive cells per mm bulbectomized olfactory epithelium (\pm SEM)	TUNEL-positive cells per mm unoperated olfactory epithelium (\pm SEM)
0	10.49 \pm 2.34	10.49 \pm 2.34
0.5	67.05* \pm 12.34	13.97 \pm 1.66
1	167.16* \pm 16.14	6.79 \pm 1.39
1.5	181.18* \pm 25.92	2.30 \pm 0.10
2	220.23* \pm 5.15	2.20 \pm 0.78
3	14.49 \pm 5.19	1.93 \pm 0.28
5	9.20 \pm 1.52	3.44 \pm 0.58
6	19.63* \pm 1.78	7.07 \pm 2.09
56	12.28* \pm 3.37	4.02 \pm 1.01
84	17.33* \pm 3.37	4.60 \pm 0.76

Adult male mice of two different strains (outbred CD-1s [Charles River] and OT-2 transgenics [kind gift of Frank Margolis, Roche Institute of Molecular Biology]) were subjected to unilateral bulbectomy, sacrificed at the indicated times, and olfactory epithelial tissue was fixed, sectioned and processed for TUNEL staining as described in Fig. 3. The mean number of TUNEL-positive cells was counted on both the bulbectomized and unoperated side for animals at each timepoint. Since no statistically significant difference was observed in the number of TUNEL-positive cells per mm of olfactory epithelium between animals of the two strains, data from both were pooled (Holcomb et al 1995). Asterisks indicate significant differences between values for the bulbectomized and unoperated sides at each timepoint (Student's *t*-test, $P < 0.02$ for all timepoints except 56 days, where $P = 0.055$). (Adapted from Holcomb et al 1995.)

While most of the cells undergoing apoptosis are ORNs, experiments combining TUNEL staining with immunocytochemistry to analyse the phenotypes of apoptotic cells have indicated that cells at all stages in the olfactory epithelium neuronal lineage undergo apoptosis at 24 hours after OBX. This includes proliferating ORN precursors, immature ORNs and mature ORNs. In contrast, other olfactory epithelium cell types not in the ORN lineage, including keratin-expressing horizontal basal cells and supporting or sustentacular cells, do not undergo apoptosis following OBX (Holcomb et al 1995). Detailed analysis of the timecourse of TUNEL staining from 12 hours to 12 weeks after OBX indicates that cell death peaks at approximately 48 hours, drops precipitously, and then attains a new steady-state level that is low, but elevated over that of controls (Table 1). Interestingly, in these long-term OBX animals the increased TUNEL staining is found primarily in mature ORNs, despite the fact that in the chronically bulbectomized olfactory epithelium the

F-2 transgenics subjected to olfactory tissue was mean number of cells per mm² olfactory epithelium at each timepoint (Adapted from

experiments analyse the stages in the s after OBX. and mature ORN lineage, sporting or Holcomb et al 12 hours to 48 hours, t is low, but g-term OBX mature ORNs, epithelium the

proportion of mature ORNs is very small (Holcomb et al 1995). Thus, olfactory epithelium neuronal cells of different types appear to undergo apoptosis differentially in the acute, versus the chronic, response to OBX. One interpretation of this result is that different factors are responsible for survival of cells at different stages in the ORN lineage, an observation that has been made for other neuronal lineages as well (e.g. Verdi & Anderson 1994).

We have used the OBX paradigm to see if p53 plays a role in trophic factor-dependent neuronal survival. These experiments have been carried out using a transgenic mouse strain in which the gene encoding p53 has been inactivated by homologous recombination (Lowe et al 1993). p53 has been characterized as a tumour suppressor gene (Michalovitz et al 1990, Kemp et al 1993) involved in negative regulation of the cell cycle (Kastan et al 1992). Its function has also been shown to be required for programmed cell death in a number of cell types, including, most interestingly to us, photoreceptor precursor cells of the mouse retina (Howes et al 1994). There is also some evidence that p53 function may be important in growth factor deprivation-induced cell death, at least in mitotically active cells: for example, loss of p53 function suppresses apoptosis in some factor-dependent leukaemic and haemopoietic cell lines (Zhu et al 1994, Gottlieb et al 1994). However, in our initial experiments we have observed no significant difference in the extent of TUNEL labelling in p53 nullizygous versus wild-type mice at any time point investigated (Table 2). This suggests that OBX-induced apoptosis in the olfactory epithelium proceeds in a p53-independent manner. It should be noted, however, that p53-dependent apoptosis has been characterized predominantly in proliferating cell populations, often following DNA damage induced by radiation or cytotoxic drugs (e.g. Lowe et al 1993), whereas its role, if any, in postmitotic cells such as neurons is unknown (Freeman et al 1994). It is possible that more than one type of cell death pathway operates in the olfactory epithelium following OBX, and that apoptosis of postmitotic ORNs is p53-independent, whereas apoptosis of ORN precursors may be dependent on p53 function. To find out whether or not this is the case, we will need to perform double-labelling experiments using a marker for mitotic cells, such as [³H]thymidine, in combination with TUNEL staining in these animals.

In order to identify factors that regulate neuronal cell survival in the olfactory epithelium, we have tested the ability of various pharmacological agents and polypeptide growth factors to promote survival and inhibit apoptosis of dissociated olfactory epithelium neuronal cells cultured from late-stage (embryonic day [E] 16.5–17.5) mouse embryos. The dissociated cell fraction consists of approximately 75% NCAM-positive ORNs and 25% NCAM-negative cells (presumptive precursors) at the time of plating; these cells are referred to as olfactory epithelium neuronal cells, or the neuronal cell fraction. When olfactory epithelium neuronal cells are plated in the absence of any added factor, they die by a process involving DNA fragmentation and virtually no

TABLE 2 Apoptosis in the olfactory epithelium of $p53^{-/-}$ mice following olfactory bulbectomy

Genotype	n	Days post-bulbectomy	TUNEL ⁺ cells per mm olfactory epithelium (\pm error)
$p53^{+/+}$	3	1	160.6 (\pm 20.4)
$p53^{-/-}$	3	1	168.0 (\pm 20.0)
$p53^{+/+}$	2	2	109.7 (\pm 11.9)
$p53^{-/-}$	4	2	130.0 (\pm 13.4)
$p53^{+/+}$	2	5	25.1 (\pm 5.5)
$p53^{+-}$	1	5	38.5 (\pm 6.3)*

Male mice (strain 129/Sv-p53; kind gift of Tyler Jacks, Massachusetts Institute of Technology) of the indicated genotypes were bulbectomized at 5–6 weeks of age and then sacrificed at 24 h, 48 h and 5 days post-bulbectomy. Olfactory epithelium was fixed by freeze-substitution, decalcified, and 12 μ m cryostat sections were cut and processed for TUNEL as described in the legend to Fig. 3. The number of TUNEL-positive cells per mm of septal olfactory epithelium was determined on the bulbectomized (given above) and contralateral (control, not shown) sides of the olfactory epithelium in a minimum of five sections for each animal tested. There was no significant difference between $p53^{-/-}$ and $p53^{+/+}$ or $p53^{+-}$ mice in the number of TUNEL-positive cells per mm of olfactory epithelium at any of the timepoints investigated. Error was calculated as the root mean square of the individual SEMs, except where indicated by asterisk (this error is the SEM of 15 sections counted for a single animal).

cells remain alive at 72 hours in culture (Holcomb et al 1995). Interestingly, when the extent of DNA fragmentation is assessed in these cultures using TUNEL staining, a close parallel is observed between the timecourse of apoptosis in cultured olfactory epithelium neuronal cells and in the olfactory epithelium *in vivo* following OBX. This suggests a similar cause of apoptosis in the two situations (Holcomb et al 1995). Indeed, explantation of embryonic olfactory epithelium into culture severs contacts between ORNs and the developing olfactory bulb, just as olfactory bulbectomy does.

We have used agents known to prevent or ameliorate apoptosis in other systems to verify that the DNA fragmentation we observe in cultured olfactory epithelium neuronal cells is indicative of this process. For example, treatment with either actinomycin D (an inhibitor of transcription) or cycloheximide (an inhibitor of translation) decreases by half the amount of TUNEL staining observed at 24 hours in olfactory epithelium neuronal cell cultures, suggesting that *de novo* gene expression is required for apoptotic death in these cells (Holcomb et al 1995, Martin et al 1988). In addition, the cell death inhibitor aurintricarboxylic acid (AT), known to prevent apoptotic death of nerve growth factor-deprived PC12 (pheochromocytoma) cells (Batistatou & Greene 1991), also decreases the level of apoptotic death in olfactory epithelium neuronal cell cultures by more than 50% (Holcomb et al 1995). Furthermore, AT promotes survival of ORNs in these cultures long after

TABLE 3 Neurotrophins promote survival of olfactory receptor neurons *in vitro*

Pharmacological agent/growth factor	Survival index (% ± SEM)
Aurintricarboxylic acid (AT, 100 µM)	100.00 ± 2.86
No growth factor	8.58 ± 2.33
8-(4-chlorophenylthio)-cAMP (1 mM)	89.57 ± 1.36
Nerve growth factor (33 ng/ml)	14.06 ± 0.68
Brain-derived neurotrophic factor (33 ng/ml)	51.94 ± 1.55
Neurotrophin 3 (33 ng/ml)	51.94 ± 2.96
Neurotrophin 5 (1 ng/ml)	38.51 ± 2.86

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Dissociated olfactory receptor neurons from E16.5–17.5 mice were grown in serum-free, defined medium as described (Calof & Lander 1991). Cultures were plated at a density of ~1.5 × 10⁴ cells/well in 96 well polystyrene trays coated with 1 mg/ml poly-D-lysine and then grown in the presence of the indicated factor for 72 hours. The total number of phase-bright, neurite-bearing cells was counted for triplicate wells in each condition (Holcomb et al 1995). Maximum percentage survival (~21% of the input cells) was observed in wells treated with AT. The survival index was calculated by dividing this maximum percentage survival by the percentage survival for each condition. Neurotrophins (obtained from Genentech Inc., South San Francisco, CA, USA) were tested at 1, 10, 33 and 100 ng/ml. Olfactory receptor neuron survival was significantly above control condition levels for all neurotrophins (except NGF) at every concentration tested; NGF gave no significant effect at any concentration tested. Asterisks mark survival indices significantly greater than that observed in the control (no growth factor) condition ($P < 0.05$, ANOVA followed by Dunnett's test for multiple comparisons against a single control). (Adapted from Holcomb et al 1995.)

morphological degeneration has ensued in control conditions: for example, when cultures are grown for 72 hours in 100 µM AT, a significant fraction (>20%) of the input cells are phase-bright and bear neurites, whereas in control cultures, less than 2% of input cells remain alive at this time (Holcomb et al 1995).

Not only ORNs, but also ORN precursors, appear to undergo apoptotic death *in vitro* as well as *in vivo*. By combining [³H]thymidine incorporation analysis with TUNEL staining and cell type-specific immunocytochemistry, we have found that a significant fraction (approximately 8%) of [³H]thymidine-labelled, NCAM-negative cells (presumptive INPs) are TUNEL-positive after 24 hours in culture. Moreover, apoptotic death of these cells is reduced by approximately 50% when cultures are grown in the presence of AT (Holcomb et al 1995). Neuron number may therefore be controlled in the olfactory epithelium not only by regulation of the birth and death of postmitotic ORNs, but also by regulation of the size of neuronal precursor cell pools. For example, polypeptide growth factors may act to control the number of neuronal precursors both by controlling the number of precursor cell divisions and by determining whether the daughter cells produced by these divisions survive to continue their development.

In this regard, it is interesting to speculate on how growth factor responsiveness may be regulated in neuronal precursor cells. Insight into this problem may come from studies of mice with deficits in genes that are essential for the developmental progression of precursor cells in different neuronal lineages. For example, we have recently begun to perform experiments analysing the olfactory epithelium in *Mash-1* 'knockout' mice. At Day 14 of embryonic development (E14), there is essentially no cell death in the olfactory epithelium of wild-type mice (A. L. Calof & M. K. Gordon, unpublished results 1995). In contrast, there is widespread cell death in the olfactory epithelium of *Mash-1*^{-/-} mice at this age (Fig. 4A). When the same sections are stained with an antibody to NCAM, it can be seen that a few NCAM-positive ORNs do develop in *Mash-1*^{-/-} mice (Fig. 4B). Interestingly, it is not these cells that are undergoing apoptosis; rather, only cells that are NCAM-negative (the majority of cells in the olfactory epithelium of these *Mash-1*^{-/-} embryos, although not in the olfactory epithelium of wild-type mice at E14) are TUNEL-positive (compare A and B). Given our current view of the ORN lineage, in which the *Mash-1*-expressing precursor cell is the progeny of the olfactory epithelium stem cell, our hypothesis is that the apoptotic cells in *Mash-1*^{-/-} embryos are stem cell daughters that are fated to express MASH-1 and then progress through the ORN lineage. In the absence of MASH-1, such cells may fail to express structural genes, such as receptors for growth factors, whose functions are essential for mediating signals that regulate neuronal precursor survival. This hypothesis predicts that the olfactory epithelium neuronal stem cell is still present in *Mash-1*^{-/-} mice, and that the TUNEL-positive cells that are observed in these embryos are recently generated precursor cells that would normally express MASH-1. These ideas are currently being tested in our laboratory.

More information is available on growth factors that regulate survival of post-mitotic ORNs. We have tested polypeptide growth factors of the neurotrophin family, the prototypical member of which is nerve growth factor (NGF), for their ability to promote survival of ORNs. In these experiments, dissociated olfactory epithelium neuronal cells are grown for 72 hours in the presence of individual neurotrophins at a variety of concentrations, and the total number of ORNs that survive is counted and compared with the number of input cells. Table 3 shows the effects of these factors on ORN survival: all neurotrophins tested, except NGF, promote survival of a fraction of ORNs at all concentrations tested (1–100 ng/ml), although no individual neurotrophin is as effective as AT. In addition, the cAMP analogue 8-(4-chlorophenylthio)-cAMP (CPT-cAMP) promotes survival of ORNs, and to about the same extent as AT (Table 3, and Holcomb et al 1995). CPT-cAMP has been shown to mimic the survival-promoting effects of NGF on sympathetic neurons (Rydel & Greene 1988), so this result is consistent with the observed positive effect of neurotrophins on promoting ORN survival. Examples of dissociated olfactory epithelium neuronal cell cultures grown for 72 hours in these different factors are shown in Fig. 5.

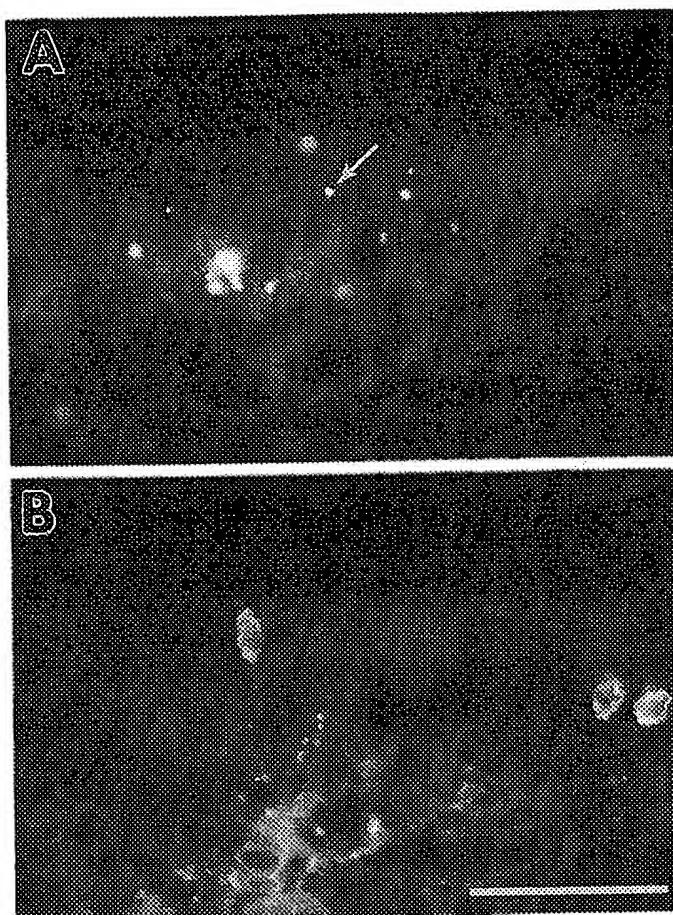
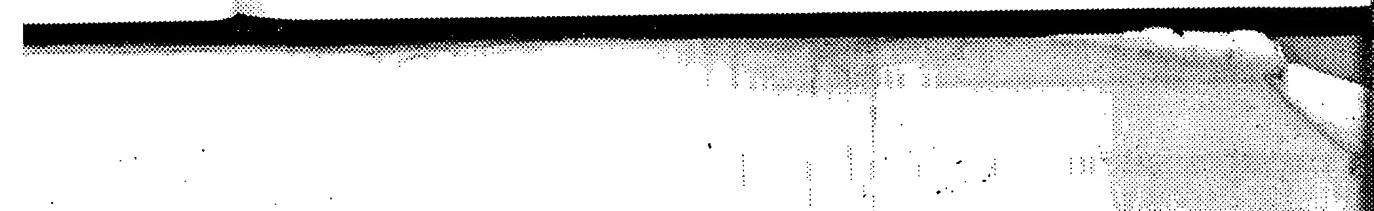


FIG. 4. Apoptosis in NCAM-negative cells of olfactory epithelium in *Mash-1*^{-/-} embryos. 12 μm cryostat section of olfactory epithelium from E14.5 *Mash-1* nullizygous embryos were processed for TUNEL staining (A, fluorescein optics) and NCAM immunoreactivity (B, rhodamine optics). Many TUNEL-positive cells can be seen in the olfactory epithelium of *Mash-1* nullizygous animals at this age (arrow, A), whereas wild-type littermates have virtually no TUNEL-positive cells in the olfactory epithelium (not shown). No overlap is seen between the NCAM-positive cells and TUNEL staining (compare A and B), suggesting that apoptosis in *Mash-1* nullizygotes occurs predominantly in NCAM-negative olfactory receptor neuron progenitor cells of the olfactory epithelium. Bar = 50 μm .

The reason for the relatively small survival-promoting effects of individual neurotrophins on ORNs is not known. One possible explanation is that only subpopulations of ORNs are responsive to these factors, a phenomenon that has been observed for sensory neurons of the trigeminal ganglion (Davies et al

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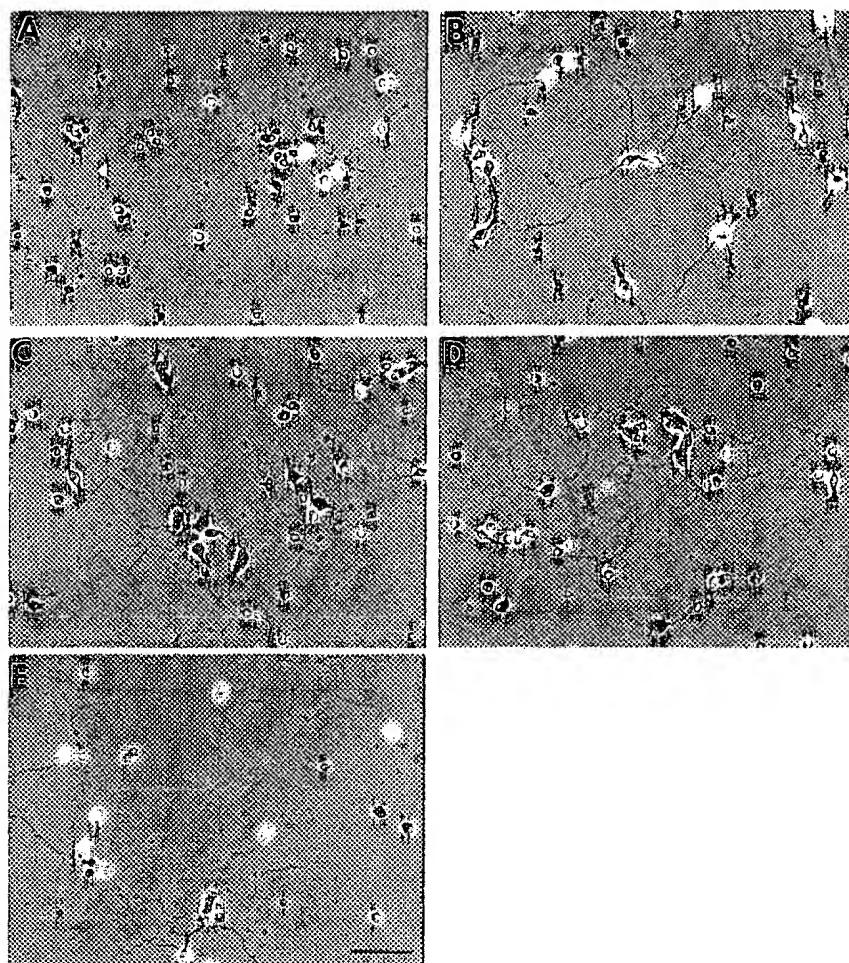


FIG. 5. Survival of cultured olfactory receptor neurons is promoted by neurotrophins. Phase photomicrographs from the experiment described in Table 3. (A) Control (no growth factor) condition at 72 hours in culture. Only fragments of degenerated cells remain. (B) 100 μ M aurintricarboxylic acid, 72 hours. Many phase-bright, neurite-bearing olfactory receptor neurons survive in this condition. (C) 33 ng/ml BDNF. (D) 10 ng/ml NT-5. (E) 1 mM 8-(4-chlorophenylthio)-cAMP. Bar = 50 μ m.

1993). Consistent with this, we have observed expression of the neurotrophin receptors, TrkB and TrkC, in fractions of ORNs found scattered throughout the epithelium in neonatal mice (Holcomb et al 1995).

We have now begun to test for the ability of neurotrophins to rescue ORNs from apoptosis induced by OBX in adult mice. In our preliminary experiments,

adult male mice have been subjected to unilateral OBX and pieces of gelfoam saturated with 10 µg of cytochrome C or 10 µg of recombinant human NT-4/5, NT-3 or NGF, placed into the cavity created by removal of the olfactory bulb. Currently, we have assessed survival times of two and five days, and have determined both the total number of TUNEL-positive cells and the thickness of the olfactory epithelium in neurotrophin-treated, versus cytochrome C-treated, animals. Thus far, no statistically significant rescue effect has been observed for any neurotrophin at either timepoint. However, it may be the case that the damage associated with olfactory bulbectomy (e.g. from axotomy) cannot be overcome by growth factor application, and that survival-promoting effects of neurotrophins may be observed only in chronically bulbectomized animals, i.e. animals allowed to survive two weeks or more post-OBX. In this situation, the great majority of apoptotic cells in the olfactory epithelium are mature ORNs (see above), so we would expect to see an increased number of mature ORNs surviving in the OBX olfactory epithelium in animals treated with effective growth factors. We are currently investigating this possibility.

Conclusions

The olfactory epithelium of the mouse has proved to be an instructive model system for gaining insights into how neuronal birth and death interact to control neuron number in the mammalian nervous system. Our studies suggest that cell number is regulated by these two processes at every stage in neuronal lineages, and that this regulation occurs during both embryonic development and regeneration in the adult.

Acknowledgements

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DISCUSSION

Greene: I'm a little unclear about the role of FGF in the olfactory epithelium. You said that it is a proliferation factor for the ORN precursors: is it also a survival factor for these cells? Do they differentiate as well?

Calof: I didn't go into all the work that we did on FGF—it is complicated. FGF is not a mitogen in the sense of altering cell cycle kinetics of the ORN

precursors. In the presence of FGF, the cells will undergo multiple divisions. We've done commitment experiments, and we find that the critical time for FGF to be present in order to allow cells to undergo a second round of division is in early G1 phase of the precursor cell cycle (DeHamer et al 1994).

Greene: If it is not present, do the cells die?

Calof: They undergo terminal differentiation into ORNs.

Greene: Are there other factors present which will promote survival or proliferation in this system?

Calof: Cell death in the cultures is not altered by FGF. The thing to keep in mind about the FGF studies is that they were performed using explant cultures, which contain all the cell types of the embryonic epithelium. It is possible that there are factors within the olfactory epithelium itself which mediate neuronal survival at these early stages of differentiation.

Raff: What proportion of cells at E14 are MASH-1 positive?

Calof: A very small proportion, but much greater than in the adult. If you count immediately after explantation into culture, about 4–5% of what we call the neuronal cell fraction expresses MASH-1 (Gordon et al 1995). This neuronal cell fraction includes precursors and differentiated neurons, but not keratin-positive basal cells or sustentacular cells.

Raff: But much greater than your estimate of stem cells?

Calof: At least by a factor of 10. The number of MASH-1-expressing cells is very low, although they are present, in the unstimulated adult olfactory epithelium. Another property of MASH-1-expressing cells that makes us think they're not true stem cells is that about 40% of MASH-1-expressing cells in adult olfactory epithelium can be labelled with a single pulse of tritiated thymidine (Gordon et al 1995). At least when you think about epidermis and corneal limbus, that would be an extraordinarily high proportion of stem cells to be labelled—typical epithelial stem cells have long cell cycles and cannot be labelled by short pulses of thymidine.

Van De Water: TrkB is expressed in the olfactory epithelium: do you also see TrkC in this epithelium, because NT-3 looked to be equally as effective as BDNF and NT-4/5 in promoting ORN survival? If it is, do you think that there are separate populations in this epithelium for TrkB and TrkC or does the same population carry both?

Calof: Similarly to TrkB, TrkC is expressed in a fraction of ORNs in the neonatal mouse olfactory epithelium (Holcomb et al 1995). At present, we do not know if they are both expressed by the same neurons.

Getchell: Your results are fascinating, particularly if we look at them from the slightly different perspective of the research on retinal ganglion cells that Albert Aguayo presented (Aguayo et al 1996, this volume). Following axotomy of the optic nerve, there was a window of opportunity of a few days where one could rescue the retinal ganglion cells, whereas with the ORNs following bulbectomy (and therefore olfactory nerve section), there is a very

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rapid onset of apoptosis. From your data, particularly in cell culture, do you see any evidence that there is a window of opportunity to rescue damaged ORNs in analogy with the retinal ganglion cells?

Calof: In Aguayo's experiments, he measured ganglion cell degeneration. If you were just looking at morphological degeneration of the epithelium (decrease in thickness), the five day window might hold—we see a gradual decrease in epithelial thickness, with the low point reached about five days after bulbectomy. In culture, we can rescue some of the ORNs—perhaps 20%. It's interesting to speculate why we can't rescue more. On the other hand, we are not able to do in this system what people can do in the sympathetic nervous system, which is to look at cell death following withdrawal of the trophic factor, because we don't yet know what the appropriate trophic factors are.

Getchell: Is there any experimental insight from the recent paper by Liu et al (1995), in which they suggested that there were soluble factors from the olfactory bulb that regulate the growth of olfactory receptor axons through the Schwann cell matrix to the olfactory bulb and might be considered to be 'maintenance factors'? In other words, if you were to take an explant of olfactory bulbar tissue and place it into your culture, would you observe a greater fibre density or rate of axon elongation toward the explant?

Calof: Probably not in these circumstances. We plate these cells on an optimized substrate to maximize axon elongation (Calof et al 1994).

Reh: One possible explanation for the longer time course of retinal ganglion cell death is that the axon stump remaining after transection is longer after optic nerve lesions than after olfactory nerve lesions. However, there is a greater intraretinal course of the ganglion cell axons.

Calof: Albert Aguayo was making the optic nerve lesions right behind the eye.

Reh: If you think about it, there's a lot of intraretinal course of those axons before you could actually make the section. In the olfactory epithelium there's not that long a distance.

Calof: I'm not so sure. In the dorsal epithelium, which is right next to the cribriform plate, the epithelium is very close to the olfactory bulb. In the lateral part of the epithelium, however, there's certainly at least a millimetre of distance between the cell bodies in the epithelium and their synapses in the olfactory bulb.

Lindsay: Have you any speculation as to why these cells turn over? What kind of signals might regulate that? Are there any manipulations that prevent turnover?

Calof: They may turn over simply because of continual environmental assault. When people have looked under controlled conditions, there's no evidence that ORNs are unusually short-lived; in fact, there is evidence for long-lived receptor neurons. It may be the case that ORN turnover would cease if we were able to protect these neurons from damage (Hinds et al 1984).

Lindsay: I guess part of my question is: is neurogenesis involved in turfing out the old cells? If you were to irradiate the epithelium, which would kill the neuronal precursors, would you still get loss of immature cells?

Calof: Do you mean pushing out the old for the new?

Lindsay: Yes.

Calof: I don't think so. In a chronically bulbectomized animal, there's an elevated level of cell death. If you look at MASH-1 expression, it is elevated to about the same degree. My interpretation of that is that the elevation of neurogenesis and MASH-1 expression is a *consequence* of the cell death, and the cell death itself is a consequence of a lack of target. In terms of going the other way—down-regulating ongoing cell death in the normal animal—I don't know yet if anything like that occurs. We've tried looking at p53 knockouts to see if there is any difference in the degree of cell death in the epithelium in these kinds of crude bulbectomy experiments. In the short term there doesn't appear to be any effect of p53. However, the role of p53 in cell death has mostly been characterized in mitotically active cells that have been damaged by chemotherapeutic agents or X-rays. It may be the case that p53 is *not* involved in trophic factor-mediated cell death in postmitotic neurons.

Barde: You showed that after bulbectomy, cell proliferation begins, as if the absence of cells were somehow a start signal. Have you looked in culture at the effect of cell density on proliferation? Could it be a membrane-mediated effect?

Calof: That's a good question. A lot of work on proliferation is done in explants where density is naturally optimized. In fact, we repeated our experiments on FGF using dissociated cells and found that density was important. If the density was too low, we were unable to observe the normal rate of proliferation in the progenitors.

Reh: Can you give us some more details about what you actually observe in the *Mash-1* knockout mice: what is the defect? Is it that they lack just terminally differentiated ORNs, or do they lack any of the other of the cell types?

Calof: We have just begun to analyse the nullizygous animals in detail. At E14–15, there is an increased level of apoptosis in the epithelium, which we think is primarily in neuronal precursors. Using antibodies to p75^{NGFR}, we have found that the Schwann or ensheathing cells of the olfactory nerve still appear to be present in the nullizygotes. This suggests to me that, if the olfactory Schwann cell and ORNs arise from a common stem cell in the epithelium or in the placode (which has been proposed), then the Schwann cell lineage diverges prior to the need for MASH-1 expression in the ORN lineage.

Getchell: Going back to Tom Reh's question about the *Mash-1* gene, in Guillemot et al's (1993) original paper on the *Mash-1* knockout, they demonstrated that during the early embryonic stages there were olfactory marker protein (OMP)-positive ORNs in the epithelium at an early embryonic stage (E13.5). But just postnatally, the vast majority of OMP-positive ORNs

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Greene: I noticed something in your data which I wanted to point out, concerning the cAMP derivative. This is at least the sixth set of neurons I know of in which a cAMP derivative turns out to be an extraordinarily effective promoter of survival. This raises the issue that one could think about targeting protein kinase A activation in neurons as a way to promote and prevent degeneration, because it looks like it's an almost universal anti-apoptotic agent for neurons.

Kessler: Going back to the clinical arena, the most common cause of olfactory loss is head trauma, where you get shearing of olfactory receptor nerve fibres in the cribriform plate. The problem there, presumably, is to orientate fibres so they are able to grow back. Do you have any ideas about what kinds of factors stimulate outgrowth of fibres and perhaps orient them?

Calof: We know that laminins will promote neurite outgrowth of ORNs. Merosin, which is a homologue of laminin, is even more effective at this than EHS (Engelbreth-Holm-Swarm) sarcoma laminin (Calof & Lander 1991, Calof et al 1994). But I think that it is going to be a very challenging problem, especially if there's any scarring, because the ORN axons must get back through the cribriform plate. I don't know how easy it's going to be to try to re-direct axon growth through this plate, since the nerve is so diffuse. David Holcomb, in my lab, has tried systemic AT in these mice once or twice following bulbectomy but, in the limited attempts he made, AT did not appear to affect cell death in the epithelium.

Kessler: AT is useful in treating ischaemia of the retina. A classic model involves compression of the eye, blocking bloodflow to the retina. In rat, AT administration (not only prior to compression but also post-compression) can almost completely rescue neurons from cell death. This is looking 12 days after the event. The eye is very accessible; we hope this will lead to another clinical trial, because it's very easy to do.

Calof: Do you have any evidence that AT was actually acting directly on the cells?

Kessler: In terms of mechanism, AT has some actions as a glutamate receptor antagonist and it has many other actions as well. Thus it is probably not possible to ascribe its actions to endonuclease inhibition. However, after retinal ischaemia you get DNA laddering and electron microscopic evidence of apoptosis, and AT prevents this apoptosis.

Greene: I don't think AT is working by endonuclease inhibition, because it's a polymer. We've tested various polymer fractions of this compound. It turns out that the bigger they get, the more effective they are, up to a molecular weight of several million. I don't think anything of this size and charge is likely to penetrate cells.

Van De Water: Has anybody looked at MASH-1 expression when the olfactory epithelium is challenged and the olfactory bulb is left intact?

Calof: No, not that I know of.

Van De Water: That would be an interesting condition to look at, when the innervation is left intact and there is severe trauma to the olfactory epithelium.

Calof: It is an interesting idea. It doesn't allow you to separate the increasing MASH-1 expression from absence of neurons, though. But what you get at the end, once you get reconstitution, might be different.

Since we're talking about the nose, what about nasal route of entry for growth factors as drugs?

Kessler: Obviously, ORNs are just about the most accessible neurons possible. A number of drugs are given nasally, so it would be a reasonable route.

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